



## THE POLYMERASE CHAIN REACTION: CLINICAL APPLICATIONS

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### 1. Introduction

In the 6 years that have elapsed since the polymerase chain reaction (PCR) technique was published it has had a major impact on medical research (E6, M8, M9, S1). Previous reviews (E3, G1, P2, W7, W9) have focused on its clinical applications in diagnosing viral and genetic diseases, and several books (E4, E5, I1) provide detailed protocols appropriate for the research laboratory.

are single strands of DNA, each approximately 20 nucleotides long. The primers are made by an automated DNA synthesizer. Specific primers can be ordered from commercial suppliers.

Each pair of primers is designed so that the nucleotide sequence of one primer is complementary to sequences flanking one end of the target DNA, whereas the other primer is complementary to the other flanking sequence. After the double-stranded target DNA is denatured into single strands, the primers hybridize to their complementary sequences flanking the target gene. The primers are oriented so that when they bind to the flanking sequences, their 3'-hydroxyl ends face the target sequence. Next, a thermostable DNA polymerase (S2) is added, and, because DNA polymerases extend DNA chains by adding deoxyribonucleoside monophosphates to the 3' end of each chain, the polymerase extends the primers, thereby making copies of the target. The extension products of each primer must be long enough so that they include the sequences complementary to the other primer.

This series of steps—DNA denaturation, primer hybridization, and DNA polymerase extension—represents a PCR cycle. Each of the three steps must be carried out at an appropriate temperature. Because the products of one cycle can serve as templates for the next, if the first cycle is followed by additional ones, more copies of the target sequence will be made. The main product of the procedure is a double-stranded DNA fragment equal in length to the sum of the lengths of the two primers and the intervening DNA. Single-stranded DNA can also serve as a template, as can RNA after a complementary DNA strand has been synthesized with a reverse transcriptase. Because the quantity of target DNA theoretically doubles with each cycle, as few as 20 cycles generates approximately a million times the amount of target sequence present initially. If only 90% of the targets are extended in each cycle, 20 cycles would yield a 375,000-fold amplification. Nontarget sequences that anneal to one primer and become extended could at most increase 20-fold in concentration during 20 cycles because the product of the first primer extension is not likely to contain the sequence region complementary to the other primer. In some cases, nonspecific binding of the other primer to the extension product of the first primer may result in exponential amplification of nontarget sequences.

Although the theory of PCR is straightforward, a major problem might be expected. In a human genome containing in excess of  $10^9$  nucleotides, a 1000-base pair target would represent only  $10^{-6}$ – $10^{-7}$  of the available DNA. The annealing of the primer to the many nontarget sequences in such complex templates, even if it occurred infrequently, would lower the purity of the target in the final product. The extent to which imperfect annealing and extension can occur depends on the temperatures during the primer-annealing and polymerase extension steps, because the specificity of primer annealing is greater at higher temperatures. The use of a heat-resistant DNA polymerase allows annealing to

be carried out at an elevated temperature, thus reducing annealing to nontarget sequences. This added selectivity now allows the experimenter to produce large amounts of virtually pure target DNA for characterization. Parameters of the reaction that affect its efficiency include the concentration of enzyme, magnesium ion, and primers. Optimization of the reaction by comprehensive titration of the components is essential for the development of a highly sensitive, reproducible, and robust assay.

## 2.2. TARGET SELECTION

For detecting an infectious disease organism one can choose among several strategies in selecting the genetic target to be amplified. Genes that provide essential functions and contain both conserved (invariant) and variable sequence regions can be targeted. Specificity can be obtained either at the amplification (primer) or detection (probe) stage. Alternatively, the target might be a virulence gene that is uniquely responsible for distinguishing pathogenic from closely related nonpathogenic strains, types, or species. Pathogen-associated targets, such as cryptic plasmid genes, surface membrane protein genes, or randomly cloned sequences, can also be used as long as specificity can be demonstrated. General guidelines for the design of primers have been described (11).

To illustrate the first strategy for detecting the bacterial pathogen that causes Lyme disease, one could target a segment of the essential, multicopy, small-subunit ribosomal RNA (rRNA) gene and design primers based on sequences that are invariant among all *Borrelia* species, but different from other bacteria outside this genus (P4). The intervening region within the amplified segment can be used to distinguish DNA amplified from *Borrelia burgdorferi* from other *Borrelia* species with a species-specific probe. Variations on this approach can be used at any level of the molecular taxonomic hierarchy, i.e., for bacterial meningitis one could use primers based on rRNA gene sequences that are identical among all eubacteria and identify specific pathogens with probes for *Haemophilus influenzae*, *Neisseria meningitidis*, etc. (L1). Alternatively, species specificity can be conferred at the amplification level by using primer-target mismatches to prevent primer extension of sequences from the nontarget organisms (K14, N1, W15). This general approach can be used for viral diseases by targeting regions of essential genes that are either conserved or pathogen specific among, for example, herpesviruses or retroviruses.

Examples of the second approach to target selection are provided by the work of Frankel and co-workers (F2) and Persing *et al.* (P3). In the former study, toxin genes and an invasion-associated locus were used to distinguish pathogenic *Escherichia coli* and *Shigella* from normal gut flora. Persing and co-workers (P3) targeted a plasmid-encoded outer-surface protein gene to identify the Lyme disease pathogen. A key factor to consider when selecting sequences for primers and